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Involvement of the Endocannabinoid System in the Development and Treatment of Breast Cancer

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14. ABSTRACT
Studies to evaluate the interaction of the synthetic cannabinoid WIN55,212-2 (WIN2) and ionizing radiation have led to preliminary results implicating a novel site of action of WIN2 in the MCF-7 breast cancer model. In previous reports, WIN2 was found to stereoselectively inhibit the growth of breast cancer cells in a dose-dependent but cannabinoid receptor-independent mechanism. Experiments described in this report evaluated antiproliferative mechanisms of WIN2 treatment both alone and in combination with ionizing radiation (IR). Evaluation of autophagy, apoptosis and necrotic mechanism failed to indicate that WIN2 promoted death of the tumor cells. Autophagy was induced both for radiation alone, WIN2 alone and WIN2 + radiation, but did not appear to be involved in the antiproliferative mechanisms of either treatment. Senescence, quantified by β-galactosidase staining, and DNA damage, quantified by γH2AX formation, confirmed that radiation treatment induced senescent growth arrest both alone and in combination with WIN2; however, WIN2 was unable to alter the magnitude of senescent growth arrest induced by IR. Comparison of γH2AX levels at 1 and 24 h also showed that WIN2 did not alter the rate of DNA repair after radiation treatment. These data assessing cell death and senescence combined with additional temporal cell count studies indicated that WIN2 induces growth arrest but not cell death. It was further concluded that augmentation of IR induced antiproliferative effects by WIN2 occur from the parallel induction of both senescent and classical growth arrest responses in the breast tumor cells.

15. SUBJECT TERMS
Breast cancer, cannabinoid receptors, cannabinoids, WIN55 212-2,

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Introduction

I am currently a predoctoral candidate at Virginia Commonwealth University working toward a Ph.D. in the Department of Pharmacology and Toxicology under the guidance of mentors Dr. David Gewirtz (primary) and Dr. Aron Lichtman (secondary). This grant is supporting my current research on a project, initiated through the Department of Defense Breast Cancer Research Program, to evaluate the utility of cannabinoids as treatments against breast cancer. A closely related goal is to determine whether the use of cannabinoids might interfere with the effectiveness of breast cancer therapies. The primary training I am receiving in addition to an in-depth understanding of current and proposed treatment of breast cancer includes the proper use of the scientific method for experimental design and technical execution at the bench. In addition, the training also heavily focuses on the communication aspects of science involving literature review, oral communication, written communication and formal presentation either through poster or slideshow based talks.

Body

Previous reports have documented our observations that both WIN55,212-2 (WIN2) and ionizing radiation (IR) have antiproliferative effects in MCF-7 cells, and when given in combination augment these antiproliferative actions. We have also demonstrated that this mechanism is likely cannabinoid receptor independent and mediated through interaction with the sphingosine-1-phosphate receptor signaling system. These findings have been published in Emery et al. (2014). The purpose of the studies described in this document was to assess the mechanism(s) mediating the antiproliferative effects of both the WIN2 and IR treatments, as well as these agents in combination.

This aspect of the project was guided by previous research, which indicates that the antiproliferative effects of radiation in the treatment of cancer are caused by DNA damage. This damage is primarily driven by radiolysis of water leading to the formation of reactive oxygen species (ROS) (Narayanan et al. 1997). This ROS formation is responsible for DNA damage expressed primarily as single and double strand breaks in DNA (Driessens et al. 2009). Radiation-induced DNA damage causes MDA-MB231 breast tumor cells to undergo apoptotic cell death demonstrated by TUNEL staining, but MCF-7 cells undergo senescent growth arrest shown by pH 6.0 dependent β-galactosidase staining (Jones et al. 2005). Artificial expression of caspase-3 in the caspase-3 deficient MCF-7 cells can induce a modest apoptotic response to radiation treatment; a robust apoptotic response requires simultaneous administration of the ATM inhibitor, caffeine and re-expression of caspase-3 in MCF-7 cells (Essmann et al. 2004). Work from Essmann et al. and Jones et al. provide evidence that the antiproliferative mechanisms of radiation are contextually dependent on the system.

Cannabinoids also have multiple antiproliferative mechanisms depending on the cannabinoid agonist used and the cancer cell type investigated. These mechanisms include autophagy (Salazar et al. 2009; Shrivastava et al. 2011, Donadelli et al. 2011, Dando et al. 2013), cell death (Giullino et al. 2009; Qamri et al. 2009; Caffarel et al. 2010) and growth arrest (Galanti et al. 2008; Park et al. 2011). However, DNA damage and senescent growth arrest have not yet been associated with cannabinoid treatment in preclinical cancer models.

Δ^9-tetrahydrocannabinol (THC) treatment-induced autophagy in U87-MG glioblastoma cells based on GFP-LC3 puncta formation and electron microscopic autophagosome imaging, and knockdown of the autophagy genes ATG1 and ATG5 resulted in increased viable cell number (Salazar et al. 2009). Other studies have linked cannabinoid-induced autophagy to a ROS mediated mechanism (Shrivastava et al. 2011, Donadelli et al. 2011, Dando et al. 2013). In MDA-MB231 cells, cannabidiol (CBD) induced autophagy based on LC3 cleavage and electron microscopic autophagosome imaging. LC3 cleavage was antagonized by the autophagy inhibitor bafilomycin and the ROS scavenger α-tocopherol (Shrivastava et al. 2011). In Panc1 cells the ROS scavenger N-acetyl-cysteine was shown to inhibit autophagy induction under synthetic cannabinoid treatment, GW405833 and arachidonoyl cyclopropamide. Autophagy was quantified by LC3 cleavage, acridine orange staining and flow cytometric quantification of autophagolysosomal staining by MDC (Donadelli et al. 2011, Dando et al. 2013).

Shrivastava et al. (2011) and Salazar et al. (2009) showed that CBD and THC, respectively, induced autophagic effects that were linked to the induction of apoptosis, and this finding that cannabinoids induced apoptosis is supported by numerous studies. ErbB2 positive tumors produced by MMTV-neu transgenic animals treated with THC and the synthetic cannabinoid JWH-133 express higher levels of cleaved caspase-3 when compared to vehicle (Caffarel et al. 2010). WIN55, 212-2 (WIN2) increased apoptotic markers in HepG2 cells with concomitant increases in the sub-G1 population, annexin V/propidium iodide (PI) positive staining and cleaved caspase 3 expression (Giullino et al. 2009). Qamri et al. (2009) showed that WIN2 and JWH-133 induce apoptosis in MDA-MB231 cells based on an increased sub-G1 population, TUNEL staining and imaging of apoptotic nuclei.

In addition to cell death, another common antiproliferative mechanism is growth inhibition. Park et al. (2011) showed that WIN2 treatment induced growth arrest in gastric cancer cells through downregulation of E2F1 and several cyclins and cyclin dependent kinases. Park et al. furthermore linked WIN2 growth arrest to inhibition of the survival protein pAKT. Park et al.'s observations support an earlier study by Galanti et al. (2008) where THC caused growth arrest via down regulation of E2F1 and cyclin A in both U251-MG and U87-MG human glioblastoma cell lines. Galanti et al.'s findings with THC in U87-MG cells are contradictory to those from Salazar et al. (2009) in which THC induced autophagic cell death in U87-MG cells, but this could be explained by Salazar's use of media containing low serum, which tends to be permissive for autophagy, and Galanti's opposite use of media containing normal serum concentrations.

As stated above, the primary goal of these studies was to assess the antiproliferative mechanism(s) for radiation and WIN2 alone or in combination. MCF-7 cells treated with the WIN2/IR combination were tested for ROS mediated antiproliferative actions, cell death (including apoptosis, necrosis, mitotic catastrophe and autophagy), changes in the DNA damage response and growth arrest (both classical and senescent).

Results

Autophagy is induced in MCF-7 cells but does not appear to be relevant to WIN2 growth inhibitory mechanism

It was hypothesized that WIN2 augmentation of the antiproliferative actions of radiation would be mediated by an autophagic mechanism based on evidence from Salazar et al. 2009 and Bristol et al. 2012. To qualitatively establish the presence of autophagy, MCF-7 cells were treated with vehicle, 12 µM WIN2, 2 Gy radiation or the combination of WIN2 + IR. At 96 h, the treated cells were stained with acridine orange (AO) and imaged. The presence of increased numbers of orange vesicles compared to vehicle treatment confirmed the promotion of autophagy in cells exposed to WIN2, IR and WIN2 + IR (**Fig 1A**).

To evaluate the potential involvement of autophagy in the antiproliferative effects of the WIN2/IR combination, cells were treated with the autophagic inhibitor chloroquine (CQ) at 5 µM in combination with the WIN2/IR combination before quantification of cell viability using trypan blue exclusion. At 96 h, CQ had no effect on viable cell number in cells treated with the vehicle, WIN2, IR or WIN2 + IR (**Fig 1B**). Acridine orange staining was used to qualitatively confirm that the 5 µM CQ treatment was properly inhibiting autophagy. Markers for blockade of autophagy in AO staining include increased vesicle number (blocked degradation) and a yellow color as opposed to orange (incomplete acidification). MCF-7 cells were treated with vehicle, adriamycin (ADR; 1 µM) or ADR + CQ before being stained with AO and imaged (**Fig 1C**). ADR induced autophagy compared to vehicle, shown by an increase in orange vesicles. CQ blocked autophagy compared to ADR, shown by an increase in yellow vesicle number. Similar results with ADR and CQ were previously demonstrated in Goehe et al. 2012. These combined results indicate that while autophagy is clearly induced, autophagy does not appear to be relevant to the antiproliferative actions of WIN2, IR or WIN2 + IR.

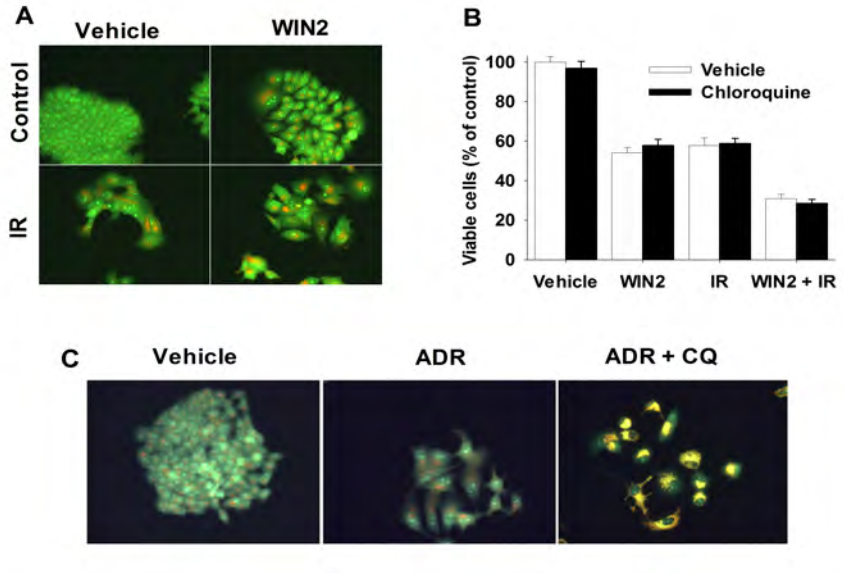

Figure 1 – Autophagy is induced by radiation and WIN2 but not involved in growth inhibition. Acridine orange staining was used to image autophagic vesicles in MCF-7 cells treated with vehicle, 12 µM WIN2, 2 Gy IR or WIN2 + IR **(A).** Cell viability was quantified using trypan blue exclusion in MCF-7 cells treated as in (A) with a co-treatment of either vehicle or 5 µM chloroquine **(B)**. Acridine orange staining was used to image autophagic vesicles in MCF-7 cells treated with vehicle, 1 µM ADR or ADR + 5 µM chloroquine **(C)**. In **(B)** data were normalized to % of control and presented as the means of 3 individual experiments ± se; no significant differences detected.

ROS do not mediate antiproliferative effects of the WIN2/IR combination

ROS have been shown to mediate cannabinoid based growth inhibition (Shrivastava et al. 2011, Donadelli et al. 2011, Dando et al. 2013, Driessens et al. 2009). To assess the involvement of ROS in the antiproliferative actions of the WIN2/IR combination, MCF-7 cells were treated with the antioxidants N-actyl-cysteine (NAC; 1 mg/ml) and glutathione (GSH; 0.5 mg/ml). NAC and GSH treatment lasted 48 h beginning 24 h before the WIN2/IR treatment (**Fig 2A-B**). 96 h after WIN2/IR were administered to MCF-7 cells neither NAC nor GSH demonstrated any ability to decrease viable cell number in MCF-7 cells. H_2O_2 (9.79 µM) was used as a positive control for ROS induced growth inhibition, and both NAC and GSH significantly protected MCF-7 cells from H_2O_2 insult at 96 h. These data demonstrate that ROS signaling does not mediate the antiproliferative actions of the WIN2/IR combination.

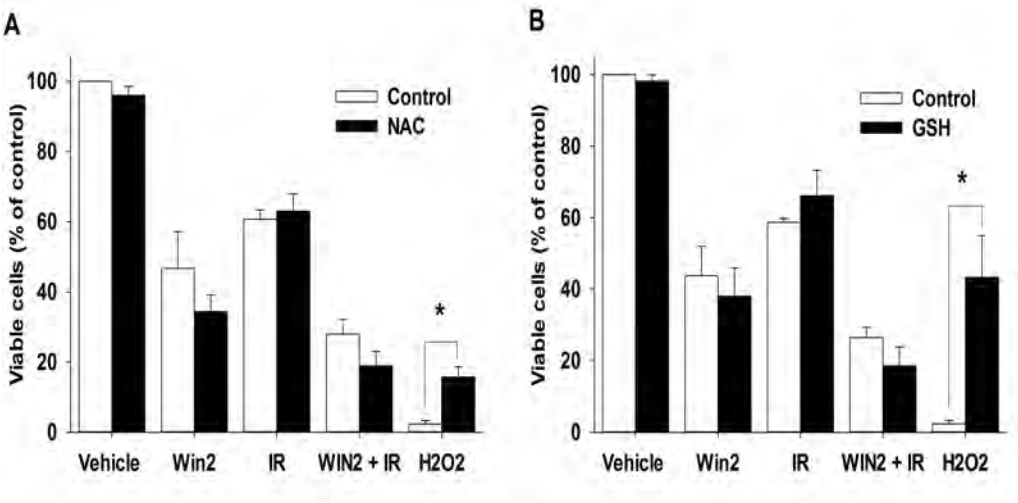

Figure 2 – ROS do not mediate the antiproliferative effects of the WIN2/IR combination. MCF-7 cells were treated with vehicle, WIN2 (12 µM), IR (2 Gy), WIN2 + IR or hydrogen peroxide (9.79 µM). Co-treatments were given of either vehicle, **(A)** NAC (1 mg/ml) or **(B)** GSH (0.5 mg/ml). Cell viability was quantified at 96 hrs using trypan blue exclusion. Data were normalized to % of control and presented as the means of 3 individual experiments ± se; *=p<0.05.

The WIN2/IR combination does not induce cell death in MCF-7 breast tumor cells

As indicated in the introduction, previous studies have documented the capacity of cannabinoids to induce apoptosis (Shrivastava et al. 2011, Salazar et al. 2009, Caffarel et al. 2010, Giullino et al. 2009, Qamri et al. 2009). In order to confirm this in our system, annexin V/PI staining was used to assess apoptosis and necrosis, respectively, at 48 h post treatment with the WIN2/IR combination (**Fig 3A**). Flow cytometric quantification showed no change in the percentage of healthy, apoptotic or necrotic cells treated with WIN2, IR or WIN2 + IR compared to vehicle. A 1 µM staurosporine treatment for 24 h was used as a positive control for apoptosis and necrosis, and this treatment produced a significant decrease in healthy cells, with significant increases in both apoptotic and necrotic cells.

To confirm the absence of cell death in MCF-7 cells treated with the WIN2/IR combination, nuclear morphology was assessed at 48, 72 and 96 h using DAPI staining (**Fig 3B**). 0.5 µM of the microtubule poison, paclitaxel, was used as a positive control for apoptotic nuclear morphology. DAPI staining was also used to screen for multinucleated cells, a marker of mitotic catastrophe (Jonathan et al. 1999). At 48, 72 and 96 h, no evidence of cell death was detected except in the positive control. Taken together, these results strongly argue that the antiproliferative effects of the WIN2/IR combination are not mediated by apoptosis, necrosis or mitotic catastrophe in MCF-7 cells.

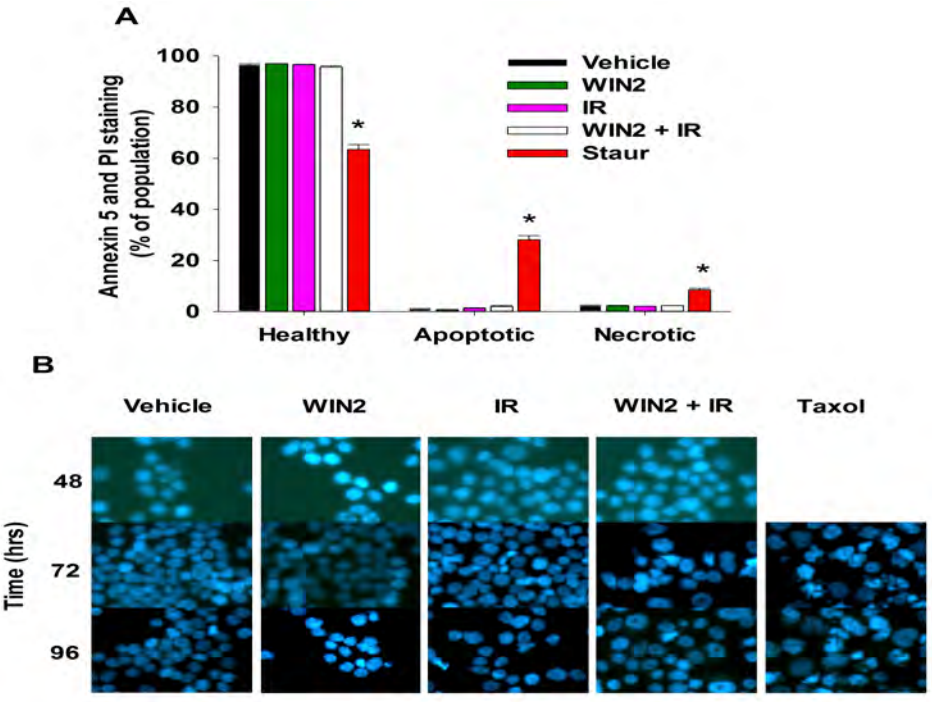

Figure 3 – Apoptosis, necrosis and mitotic catastrophe are not involved in the antiproliferative actions of the WIN2/IR combination. MCF-7 cells were treated with vehicle, WIN2 (12 µM), IR (2 Gy) or WIN2 + IR. Staurosporine (1 µM) and Paclitaxel (1 µM) were used as positive controls. **(A)** Flow cytometry was used to quantify annexin V and PI staining at 48 hrs. **(B)** Dapi staining was used to assess nuclear morphology at 40x magnification. Data normalized to % of population in **(A)**; data presented reflect the means of 3-4 individual experiments ± se; *p<0.05 vs vehicle.

Temporal effects of the WIN2/IR combination in breast cancer cells

The absence of evidence for a cell death mechanism, lead us to predict that growth inhibition was likely mediating the antiproliferative actions of the WIN2/IR combination. To test this hypothesis, trypan blue was used to assess cell viability at 24, 48, 72 and 96 h in MCF-7 cells treated with the WIN2/IR combination (**Fig 4**). WIN2, IR and WIN2 + IR were all significantly different from vehicle at 48, 72 and 96 h, which confirmed the growth inhibition hypothesis. Decreases in doubling times after treatment emphasize the presence of growth inhibition. Doubling time presented in hrs (mean±se): vehicle – 27.7±2.6, WIN2 – 44.6±7.7, IR – 36.8±4.9 and WIN2 + IR – 68.3±9.8.

A cell viability time course was also evaluated in MDA-MB231 breast tumor cells exposed to the WIN2/IR combination treatment (**Fig 5**). MDA-MB231 cells were treated with 15 µM WIN2 and 2 Gy radiation. Statistical comparisons showed that growth inhibition was detected as early as 48 h in the WIN2 and WIN2 + IR groups but by 72 and 96 h, all treatment groups showed significant growth inhibition compared to vehicle. These results support those reported using MCF-7 cells.

Different than MCF-7 or MDA-MB231 cells, 4T1 cell assessment of temporal effects indicates evidence for cell death with the WIN2/IR combination. 4T1 cells were treated with 30 µM WIN2 and 8 Gy IR before assessment at 24 and 48 h (**Fig 6**). Antiproliferative action was detected in the IR and WIN2 + IR group at 24 h and all treatment groups at 48 h when compared to vehicle. Interestingly, significant decreases in viable cell number compared to time 0 showed evidence for cell death in the IR treatment at 24 h and the WIN2 + IR treatment at 24 and 48 h. This suggests that WIN2 and IR may be interacting via a cytotoxic mechanism in 4T1 cells.

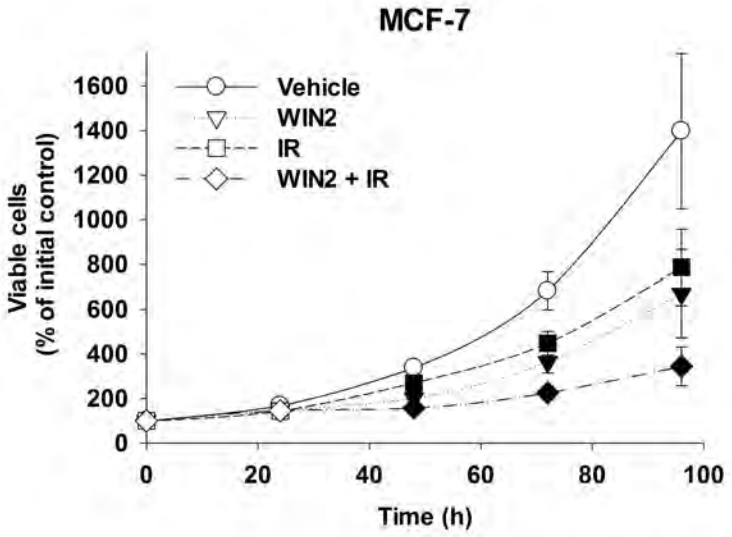

Figure 4 – Temporal effects of WIN2 and IR combination in MCF-7 breast cancer cells. MCF-7 cells were treated with vehicle, WIN2 (12 µM), IR (2 Gy) or WIN2 + IR treatments. Viable cell number was monitored over a period of 96 h using the trypan blue exlusion assay. Data presented reflect the means of 5 individual experiments ± se. Darkened symbols = $p<0.05$ vs vehicle within time points.

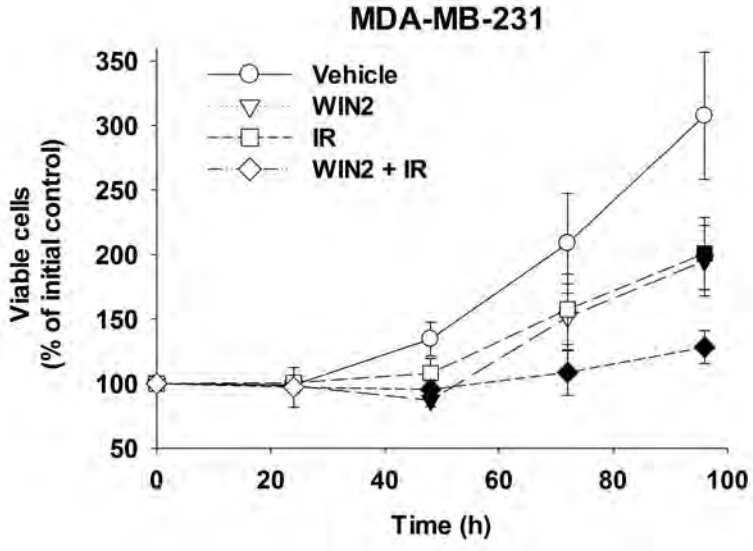

Figure 5 – Temporal effects of WIN2 and IR combination in MDA-MB231 breast cancer cells. MDA-MB231 cells were treated with vehicle, WIN2 (15 µM), IR (2 Gy) or WIN2 + IR treatments. Viable cell number was monitored over a period of 96 h using the trypan blue exlusion assay. Data presented reflect the means of 5 individual experiments ± se. Darkened symbols = $p<0.05$ vs vehicle within time points.

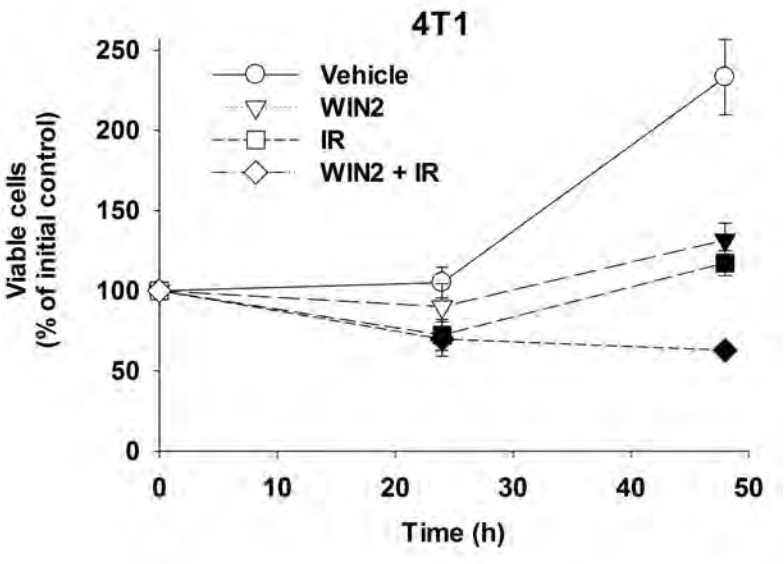

Figure 6 – Temporal effects of WIN2 and IR combination in 4T1 breast cancer cells. 4T1 cells were treated with vehicle, WIN2 (30 µM), IR (8 Gy) or WIN2 + IR treatments. Viable cell number was monitored over a period of 48 using the trypan blue exlusion assay. Data presented reflect the means of 5 individual experiments ± se. Darkened symbols = $p<0.05$ vs vehicle within time points.

Radiation but not WIN2 induces DNA damage in breast cancer cells

Antiproliferative effects of IR have been linked to the induction of DNA damage (Narayanan et al. 1997). γH2AX is a protein recruited to DNA repair complexes that is rapidly degraded after the completion of DNA repair. As a result, it is used to monitor the DNA repair response process (Rogakou et al. 1999). Changes in γH2AX expression after radiation treatment were used to assess the potential influence of the WIN2/IR combination on DNA damage induction (1 h) and repair (24 h) in MCF-7, MDA-MB-231 and 4T1 cells (**Fig 7-9**).

In MCF-7 cells, radiation significantly increased γH2AX expression at 1 h, while WIN2 alone had no effect on γH2AX. Interaction comparison indicates that WIN2 had no effect on the level of γH2AX induction by IR at 1 h, which indicates WIN2 had no effect on DNA damage induction either alone or in combination with IR. By 96 h γH2AX levels in all treatments had returned to baseline levels relative to vehicle demonstrating that WIN2 had no effect on the DNA repair process in MCF-7 cells.

In MDA-MB231 and 4T1 cells radiation significantly increased γH2AX expression at 1 h and WIN2 alone had no effect. WIN2 + IR displayed no greater induction of γH2AX at 1 h than IR alone indicating WIN2 had no effect on DNA damage induction either alone or in combination with IR. By 96 h γH2AX levels in WIN2 alone and IR alone treatments had returned to baseline levels, but γH2AX in the WIN2 + IR treatment remained significantly different from vehicle. Nevertheless, statistical comparisons showed no significant interaction between the WIN2 and IR treatments indicating no presence of augmentation. These results confirm that like in MCF-7 cells, WIN2 had no effect on the induction or repair of DNA damage in MDA-MB231 or 4T1 cells.

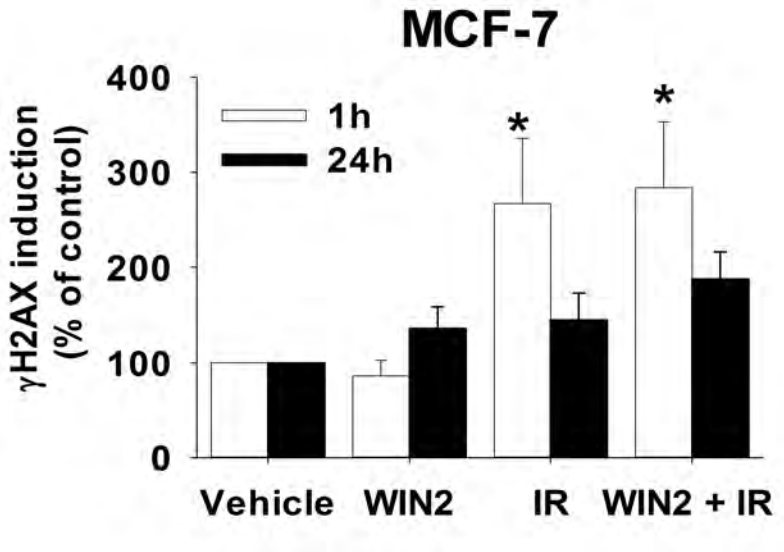

Figure 7 – DNA damage and repair in breast cancer cells treated with WIN2 and radiation. MCF-7 figure 4. γH2AX formation analyzed by flow cytometry at 1 h and 24 h after drug treatment. Data were normalized to percent of control; data presented reflect the means of 3-5 individual experiments ± se; *$p<0.05$ vs vehicle.

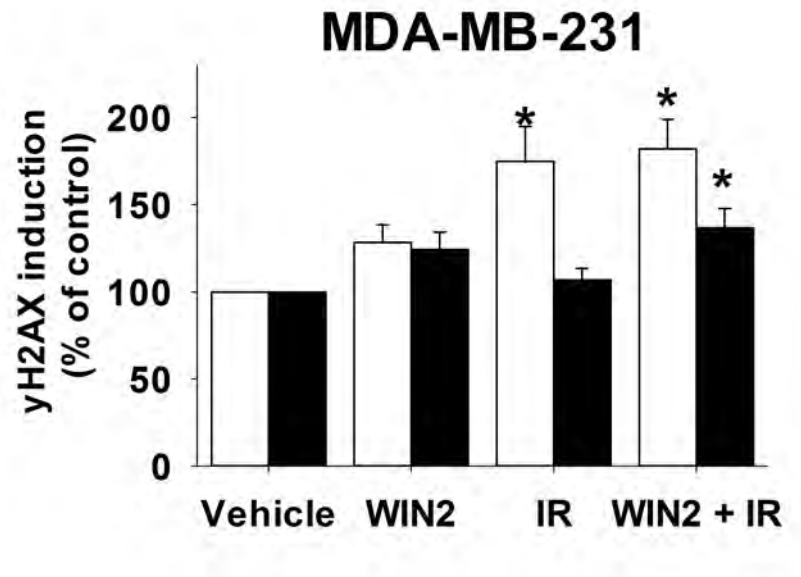

Figure 8 – DNA damage and repair in breast cancer cells treated with WIN2 and radiation. MDA-MB231 cells were treated as in figure 5. γH2AX formation analyzed by flow cytometry at 1 h and 24 h after drug treatment. Data were normalized to percent of control; data presented reflect the means of 3-5 individual experiments ± se; *p<0.05 vs vehicle.

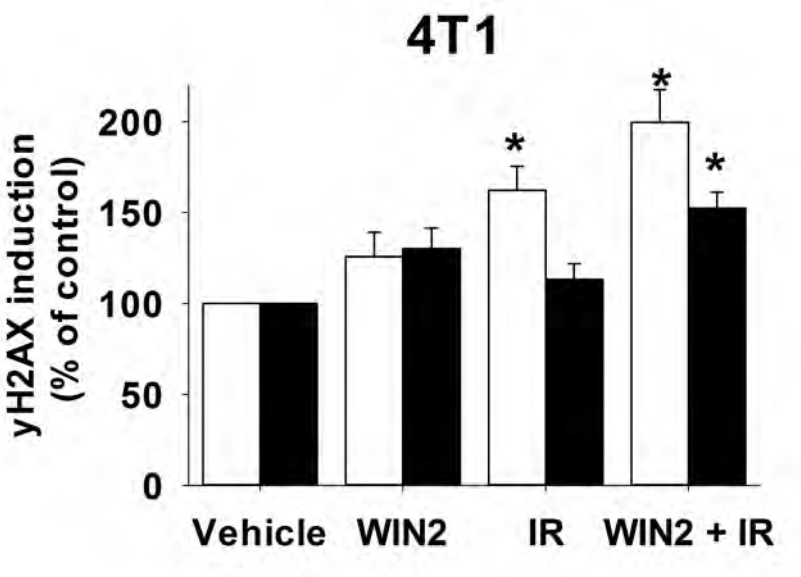

Figure 9 – DNA damage and repair in breast cancer cells treated with WIN2 and radiation. 4T1 cells were treated as in figure 6. γH2AX formation analyzed by flow cytometry at 1 h and 24 h after drug treatment. Data were normalized to percent of control; data presented reflect the means of 3-5 individual experiments ± se; *p<0.05 vs vehicle.

Radiation but not WIN2 induces senescence in MCF-7 cells

Jones et al. (2005) established that radiation treatments induced growth arrest via senescence in MCF-7 cells. To test the induction of senescence, the β-galactosidase assay was used to quantify cells treated with vehicle, WIN2 (12 μM), IR (2 Gy) or WIN2 + IR (**Fig 10A-B)**. At 96 h, radiation significantly induced senescence, WIN2 had no ability to induce senescence and interaction comparisons confirm WIN2 had no significant effect on the level of radiation-induced senescence. These studies confirm previous reports that radiation inhibits growth via senescence (Jones et al. 2005), and in the absence of senescence it can be concluded that growth inhibition after WIN2 treatment is classical growth arrest.

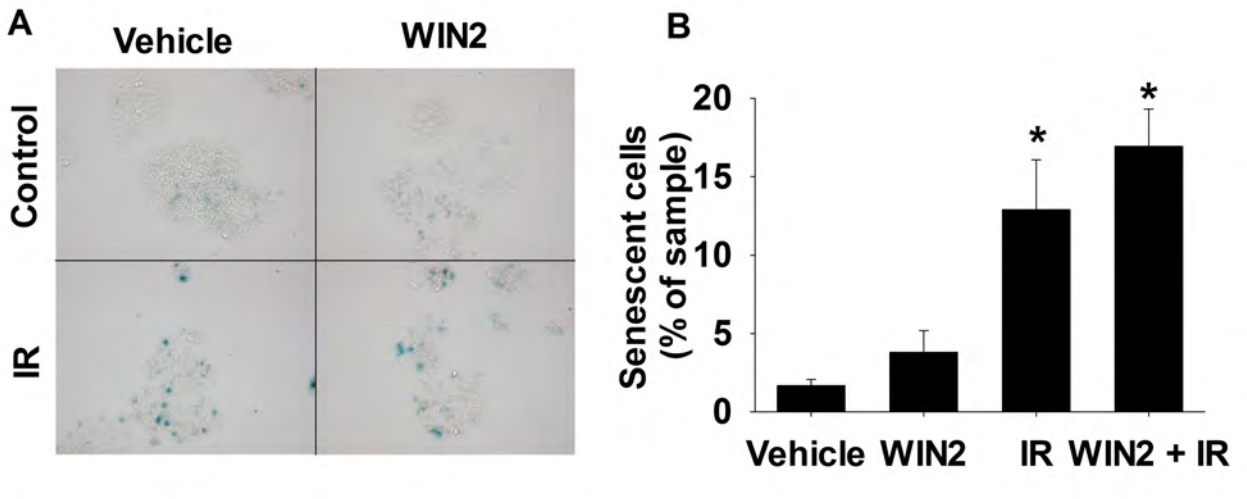

Figure 10 – Senescence induction by radiation ± WIN2. MCF-7 cells were treated with vehicle, WIN2 (12μM), (2Gy) radiation or WIN2 + radiation. **(A)** Representative images of β-galactosidase stained cells. **(B)** Quantification of β-galactosidase activity 96 h after drug treatment. Data were normalized to % of sample in **(B)**; data presented reflect the means of 3 individual experiments $\pm$ se; *$p<0.05$ vs vehicle.

Key research accomplishments

- Autophagy is present under WIN2, IR and WIN2 + IR treatments
    - Confirmed by acridine orange staining
- Autophagy is not mediating the antiproliferative actions of WIN2, IR or WIN2 + IR
    - Confirmed by chloroquine treatment in MCF-7 cells
- Cell death is not mediating the antiproliferative effects of WIN2, IR or WIN2 + IR
    - Confirmed by Annexin V/PI and DAPI staining
- Radiation induces DNA damage alone or in the presence of WIN2, but WIN2 does not induce DNA damage nor does it alter radiations magnitude of DNA damage
    - Confirmed by γH2AX
- Radiation induces senescence alone or in combination with WIN2, but senescence is not induced or altered by the presence of WIN2
    - Confirmed by β–galactosidase staining
- WIN2 induces classical growth arrest
    - Confirmed by temporal cell viability studies and the absence of cell death and senescence
- The augmentation seen with the WIN2 + IR combination comes form parallel induction of both senescent growth arrest and classical growth arrest.

Reportable outcomes

Degree completed

- PhD – this work was included in the dissertation for the defense of PhD completed in January 2014

Publications

- **Emery SM,** Alotaibi MR, Tao Q, Selley DE, Lichtman AH, Gewirtz DA. Combined antiproliferative effects of the aminoalkylindole WIN55,212-2 and radiation in breast cancer cells. (2014) J Pharmacol Exp Ther 348(2):293-302.

Abstracts submitted to

- Carolina Cannabinoid Collaborative Conference
- Virginia Academy of Science
- American Association of Cancer Research
- Pharmacology and Toxicology Research Retreat
- International Cannabinoid Research Symposium
- Carolina Cannabinoid Collaborative Conference

Presentations

- Carolina Cannabinoid Collaborative Conference – Presentation – "Enhanced Antiproliferative Actions of Combined Radiation and WIN55,212-2 on MCF-7 Breast Cancer Cells: Exploration of a Mechanism of Action" – Greenville, North Carolina
- Virginia Academy of Science – Presentation – "The Interaction Between WIN55,212-2 and Radiation on Inhibiting the Growth of Breast Cancer Cells" – Norfolk State University, Norfolk, Virginia
- American Association of Cancer Research – Poster – "The Cannabinoid WIN55, 212-2 Enhances the Response of Breast Cancer Cells to Radiation" – Chicago, Illinois
- Pharmacology and Toxicology Research Retreat – Poster – "Combining Cannabinoids and Radiation in Breast Cancer" – Williamsburg, Virginia
- Watts Day Presentation – Poster – "Combining Cannabinoids and Radiation in Breast Cancer" – Virginia Commonwealth University, Richmond, Virginia
- Massey Cancer Center Research Retreat – Poster – "Combining Cannabinoids and Radiation in Breast Cancer" – Virginia Commonwealth University, Richmond, Virginia
- Department of Pharmacology and Toxicology – Student Seminar Series – "The Interaction Between WIN55,212-2 and Radiation on Breast Cancer" – Virginia Commonwealth University, Richmond, Virginia
- Pharmacology and Toxicology Research Retreat – Poster – "Combining Cannabinoids and Radiation in Breast Cancer" – Virginia Commonwealth University, Richmond, Virginia
- Era of Hope – Poster – "Role of the Endogenous Cannabinoid System in a Murine Model of Breast Cancer" – Washington DC
- Virginia Academy of Science – Presentation – "Combining Cannabinoids and Radiation Therapy in Breast Cancer" – University of Richmond, Richmond, Virginia
- 7th Annual Women's Health Research Day at VCU – Poster – "Combining Cannabinoids and Radiation Therapy in Breast Cancer" – Virginia Commonwealth University, Richmond, Virginia
- Department of Pharmacology and Toxicology – Student Seminar Series – "Cannabinoids and Cancer Therapy" – Virginia Commonwealth University, Richmond, Virginia
- International Cannabinoid Research Symposium – Poster – "Combining Cannabinoids and Radiation Therapy in Breast Cancer" – Chicago, Illinois

Conclusion

It was originally hypothesized that WIN2 would augment the impact of radiation in breast tumor cells through an autophagic mechanism based on observations from Salazar et al. 2009 and Bristol et al. 2012. In the studies presented above WIN2, IR and WIN2 + IR were capable of inducing autophagy in MCF-7 cells; however based on the inability of CQ to alter MCF-7 response to WIN2, IR or WIN2 + IR, it can be concluded that autophagy is not relevant to the antiproliferative mechanisms for these three treatments, and furthermore autophagy is not involved in WIN2 augmentation of radiation.

One discrepancy regarding autophagy induction in these studies and in the work of Bristol et al. (2012) is that in Bristol et al., CQ enhanced the antiproliferative effects of radiation in MCF-7 cells. A CQ induced enhancement of the antiproliferative effects of radiation was not observed in the studies presented above. This might be explained by the different doses of radiation used between the studies (1x2 Gy here; 5x2 Gy in Bristol et al.), which could lead to different autophagic mechanisms. In fact, Bristol et al. observed that autophagy had different mechanisms of action depending on the conditions of the system, which suggests that the relatively low radiation dose of 2 Gy used in the current work simply may not have been sufficient to induce the protective autophagy that was reported in Bristol et al. 2012.

Previous studies have connected the antiproliferative actions of cannabinoids to ROS induced autophagy (Shrivastava et al. 2011, Donadelli et al. 2011, Dando et al. 2013). As autophagy was apparently not directly relevant to the antiproliferative mechanism of the WIN2/IR combination, and the antioxidants NAC and GSH were unable to rescue MCF-7 cells from the antiproliferative actions of the WIN2, IR or the WIN2 + IR combination, it can be concluded that ROS do not mediate the antiproliferative effects of WIN2, IR or WIN2 + IR. On the other hand, it is well documented that ROS mediate the DNA damaging effects of radiation therapy (Driessens et al. 2009), and NAC or GSH failed to protect MCF-7 cells from radiation might be confusing. However, this discrepancy can be explained by a report that has shown increased GSH levels from NAC treatment were unable to protect lung tumor cells from the antiproliferative effects of ionizing radiation (Wanamarta et al. 1998), and another study showed that overexpressing glutathione peroxidase in MCF-7 cells protected the cells from H_2O_2 treatment but not radiation treatment (Liebmann et al. 1995). Based on the Wanamarta et al. and Liebmann et al. studies, it is understandable that NAC and GSH can protect MCF-7 cells from the antiproliferative effects of H_2O_2 but not from radiation.

Annexin V and PI staining as well as DAPI nuclear staining showed that WIN2, IR and WIN2 + IR fail to induce apoptosis, necrosis and mitotic catastrophe in MCF-7 cells. The absence of these three cell death mechanisms as well as evidence that autophagy is not associated with the antiproliferative action of the WIN2/IR combination strongly argue that growth inhibition and not cell death is mediating the antiproliferative actions of WIN2, IR and WIN2 + IR. A growth inhibition hypothesis is also evident in the time course studies in MCF-7 cells treated with WIN2, IR and WIN2 + IR, as well as reports from the literature where WIN2 induced growth arrest as its primary mechanism of action (Park et al. 2011). Therefore, it appears likely that augmentation of IR induced growth inhibition by WIN2 is expressed in at least one of two ways, which is by the augmentation of one growth inhibitory pathway or the activation of two parallel growth inhibitory pathways. The two primary growth inhibitory pathways are senescence, involving the activation of a specific signalling process, or classical growth arrest, which is a more broad suppression of mitogenic signals (Blagosklonny et al. 2003). Previous studies have demonstrated using the β-galactosidase assay that radiation-induced growth inhibition is expressed as senescent growth arrest in MCF-7 cells (Jones et al. 2005). WIN2, however, failed to induce senescence or to augment the induction of senescence by radiation in MCF-7 cells. These combined observations demonstrate that growth inhibition by WIN2 is mediated by classical growth arrest, either expressed as cell cycle arrest or growth delay, and the augmentation of IR treatment by WIN2 is expressed as two parallel pathways of growth inhibition, growth arrest and senescence.

DNA damage is believed to be responsible for the induction of senescence by radiation in MCF-7 cells (Jones et al. 2005). Tracking the DNA damage response by monitoring the induction and decline of γH2AX (Rogakou et al. 1999) confirmed the presence of DNA damage by radiation in MCF-7 cells. These experiments also confirmed that WIN2 has no ability to enhance induction of DNA damage, induce DNA damage or alter the DNA repair process. Lack of interaction between WIN2 and IR treatment on γH2AX induction was also demonstrated in the MDA-MB231 and 4T1 cells. The absence of interaction between WIN2 and IR in the DNA damage response pathway supports the hypothesis that WIN2 and IR are acting via parallel mechanisms of growth inhibition rather than by a common pathway.

The conclusion of parallel growth inhibitory pathways cannot be extended to the MDA-MB231 and 4T1 cells for multiple reasons. First, MDA-MB321 cells have previously been shown to respond to radiation therapy via an apoptotic mechanism and not senescence (Jones et al. 2005). Second, the time course viability studies for 4T1 cells shows that at 24 h radiation and WIN2 + radiation significantly decrease viable cell number compared to the 0 h controls, indicating cell death as opposed to growth arrest. These lines of evidence supporting radiation-induced cell death mechanisms in MDA-MB231 and 4T1 cells do, however, allow for the conclusion that the interaction of WIN2 and IR in MDA-MB231 and 4T1

cells is different than for MCF-7 cells. If the mechanism of growth inhibition for WIN2 in MCF-7 cells extends to MDA-MB231 and 4T1, it would be logical to hypothesize that radiation is inducing cell death in a percentage of the population and WIN2 is inducing growth arrest in the remainder. Evaluating this hypothesis of parallel mechanisms of growth inhibition and cell death in MDA-MB231 and 4T1 cells in future studies could provide a crucial insight about the interaction between WIN2 and IR in MDA-MB231 and 4T1 cells.

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